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Identification of regulatory and cargo proteins of endosomal and secretory pathways in *Arabidopsis thaliana* by proteomic dissection.

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ABC – Ammonium Bicarbonate

AGC – Automatic Gain Control

ARF-GEFs – ADP Ribosylation Factor – GTP Exchange Factor

CID – Collision Induced Dissociation

CME – Clathrin Mediated Endocytosis

COG – Conserved Oligomeric Golgi

COP – Coat Protein

DTT – Dithiothreitol

EE – Early Endosome

ENTH – Epsin N terminal Homology

ER –Endoplasmic Reticulum

GARP – Golgi Associated Retrograde Protein

GDF – GDI Dissociation Factor

GDI – GDP Dissociation Inhibitor

LE – Late Endosomes

LTQ – Linear Ion Trap

MS- Mass Spectrometry

MVB – Multi-vesicular body

PM – Plasma Membrane

PVC – pre-vacuolar compartments

TBST – Tris Buffered Saline 0.1% (w/v) Tween 20

TGN – *trans*-Golgi Network

Y2H – Yeast two Hybrid

SUMMARY

The cell's endomembranes comprise an intricate, highly dynamic and well-organised system. In plants the proteins that regulate function of the various endomembrane compartments and their cargo remain largely unknown. Our aim was to dissect subcellular trafficking routes by enriching for partially overlapping subpopulations of endosomal proteomes associated with endomembrane markers. We selected RABD2a/ARA5, RABF2b/ARA7, RABF1/ARA6, and RABG3f as markers for combinations of the Golgi, *trans*-Golgi Network (TGN), Early Endosomes (EE), secretory vesicles, Late Endosomes (LE), Multivesicular Bodies (MVB) and the tonoplast. As comparisons we used Golgi transport 1 (GOT1), which localises to the Golgi, Clathrin Light Chain 2 (CLC2) labelling Clathrin-coated vesicles and pits and the Vesicle Associated Membrane Protein 711 (VAMP711) present at the tonoplast. We developed an easy-to-use method by refining published protocols based on affinity purification of fluorescent fusion constructs to these seven subcellular marker proteins in *Arabidopsis thaliana* seedlings. We present a total of 433 proteins, only five of which were shared among all enrichments, while many proteins were common between endomembrane compartments of the same trafficking route. Approximately half, 251 proteins, were assigned to one enrichment only. Our dataset contains known regulators of endosome functions including small GTPases, SNARES and tethering complexes. We identify known cargo proteins such as PIN3, PEN3, CESA and recently defined complexes such as TPLATE. The sub-cellular localisation of two GTPase regulators predicted from our enrichments was validated using live-cell imaging. This is the first proteomic data set to discriminate between such highly overlapping endomembrane compartments in plants and can be used as a general proteomic resource to predict the localisation of proteins, identify the components of regulatory complexes and provides a useful tool for the identification of new protein markers of the endomembrane system.

INTRODUCTION

Membrane compartmentalization is an essential mechanism for eukaryotic life, by which cells separate and control biological processes. Plant growth, development and adaptation to biotic and abiotic stress all rely on the highly dynamic endomembrane system, yet we know comparatively little about the proteins regulating these dynamic trafficking events. The plasma membrane (PM) provides the main interface between the cell and its environment; mediating the transfer of material to and from the cell and is a primary site for perception of external signals. Transmembrane proteins are synthesised in the endoplasmic reticulum (ER) and trafficked to the PM via the Golgi, although there are other secretory routes for soluble cargo (discussed in [1-4]). *Post*-Golgi trafficking is the main route by which newly synthesized transmembrane proteins and cell wall glycans are delivered to the PM. In plants, secretory and endocytic traffic converge at the *trans*-Golgi network (TGN), which also functions as an early endosome (EE). Multi-vesicular bodies (MVBs) are the other main endosomal compartment in plants and serve as pre-vacuolar compartments (PVCs) or late endosomes (LE) destined for vacuolar degradation (reviewed [1, 5, 6]).

Recycling and sorting of plasma membrane proteins is essential for generating the polar localisation of auxin efflux transporters (discussed in [7]), formation of the cell plate during cell division [8-10] [11], in defence such as localised deposition of papilla reviewed in [12, 13]. Furthermore, the subcellular localisation of transporters and receptors is dynamically regulated. For example, the boron transporter (BOR1) exhibits polar localisation and is internalised and degraded under conditions of high boron to reduce toxicity [14, 15]. Similarly the Receptor Like Kinases (RLKs) Flagellin Sensing 2 (FLS2) and Brassinosteroid Insensitive 1 (BRI1), important transmembrane receptors in antibacterial immunity and plant development, respectively, are constitutively endocytosed and recycled to the PM [16-18]. Both receptors and transporters are also cargoes of the LE/MVB trafficking route [16], and are probably sorted to the vacuole for degradation [19, 20]. Importantly, FLS2 trafficking via the recycling endocytic or the late endocytic route depends on its activation status, inactive receptors are recycled whilst ligand-activated receptors are sorted to the late endosomal pathway [16]. Similarly, the polar sorting of auxin efflux transporters depends on their phosphorylation status [21]. These observations illustrate that membrane

compartmentalization underpins important aspects of plant cell biology and has initiated a quest toward a better understanding of the endomembrane compartments, and the routes and mechanisms by which cargo is trafficked and sorted within the cell.

Membrane trafficking within the cell requires complex machinery consisting of a plethora of coat and adaptor proteins, small GTPases, targeting, tethering and scission factors (reviewed [22, 23]). Homologues of some animal and yeast and endomembrane regulators have been identified in plants but the localisation and function of many of these remain to be characterised. For example, members of the RAB GTPase family have been shown to have markedly different roles and localisations in plants compared to their animal and yeast homologs [24]. Therefore acquiring localisation data for tethering complexes and other regulators in plant systems is essential. In *Arabidopsis thaliana* some of these proteins have been developed as useful probes to visualize the different endomembrane compartments by fusion with fluorescent reporters [9, 25-27]. These include regulators of trafficking events such as RAB GTPases that are molecular switches responsible for the assembly of tethering and docking complexes and compartment identity. RAB proteins are widely used markers of endomembrane compartments, for example RABD2a/ARA5 labels the Golgi and TGN/EE as well as *post*-Golgi vesicles [4, 24, 26, 28], RABF2b/ARA7 localises to TGN/EE and LE [25], RABF1/ARA6 is a marker of the LE/MVB vesicles [25, 29], and RABG3f localises to MVBs and the tonoplast [26, 30].

Fluorescent-tagged marker lines for the live-cell imaging of plant cells have been invaluable in defining the location of proteins within and between organelles and endomembrane compartments [26]. However, microscopic investigation of membrane trafficking is limited by throughput, as only few proteins can be studied simultaneously. A powerful approach to large-scale identification of proteins in endomembrane compartments is through subcellular fractionation based on physical properties to directly isolate or enrich for the subcellular compartment of interest. Subcellular fractionation-based proteomics have been successfully used to decipher the steady state and cargo proteomes of, including but not limited to, the ER, the vacuole, PM, mitochondria and chloroplasts, and smaller vesicle-like compartments such as peroxisomes and Golgi [31-41]. However, the

smaller, transitory, vesicles of the secretory and endocytic pathways, have proved challenging to purify for reliable proteomic analysis. To overcome this, affinity purification of vesicles was established in animal cells [42, 43] and recently successfully applied in plants in combination with subcellular fractionation. Affinity purification and Mass Spectrometry (MS) of Syntaxin of Plants 61 (SYP61)-positive TGN/EE compartments identified 145 proteins specifically enriched in [44], while affinity isolation of VHA-a1-GFP (Vacuolar H^+ ATPase A1) identified 105 proteins associated with the TGN/EE [45]. The VHA-A1 affinity purification data was then further refined using density gradient centrifugation to differentiate cargo and steady state proteins [45].

We have further explored affinity purification of fluorescent-tagged markers localizing to defined compartments to identify proteins associated with trafficking. Our motivation was to dissect the trafficking routes by enriching for partially overlapping subpopulations of endosomal proteomes associated with small GTPases in the RAB family. We selected RABD2a/ARA5, RABF2b/ARA7, RABF1/ARA6, and RABG3f as markers for Golgi/TGN/EE/secretory vesicles, LE/MVB compartments, LE/MVB compartments and LE/MVB/tonoplast, respectively. Additionally, we used Golgi transport 1 (GOT1), which localises to the Golgi, Clathrin Light Chain 2 (CLC2) labelling Clathrin-coated vesicles (CCVs) and pits and the Vesicle Associated Membrane Protein 711 (VAMP711) present at the tonoplast [26, 27, 29, 46, 47] as comparisons. Our objective was to identify transient cargo proteins, tethers and docking factors associated with dynamic subdomains of the endomembrane system, to supplement better-characterised 'steady-state' components and to identify components of recycling and vacuolar trafficking pathways.

EXPERIMENTAL PROCEDURES

Plant materials - The following *A. thaliana* lines were used in this study (accession Columbia-0 if not otherwise stated): (Wave lines 1, 5, 29, 9 and 18) pUB:mCherry and YFP, YFP-RABG3f, YFP-RABD2a/ARA5, YFP-VAMP711, YFP-GOT1 [26], RABF1/ARA6-RFP, RABF2b/RFP-ARA7 (provided by K. Schumacher, Heidelberg, Germany), p35S:CLC2-GFP in WS-2 (Wassilewskija) background (provided by S. Bednarek, Madison, WI, USA). For protein extraction and affinity purification, 0.1 g of *A. thaliana* seed for all constructs were grown in sterile 200 ml of Murashige and Skoog medium

at 22 °C, 16 hours light, shaken at 120 rpm. For microprojectile bombardment assays, four-six weeks old *A. thaliana* plants were grown on soil under controlled conditions of 22 °C, 12 hours light, 60% humidity.

Protein Extraction and Affinity Purification – For all constructs, 30 – 50 g of eight day old seedlings of *A. thaliana* seedlings were harvested and frozen in liquid nitrogen and ground with a pestle and mortar. Protein extraction buffer (150 mM Na-HEPES pH7.5, 10 mM EDTA, 10 mM EGTA, 17.5% (w/v) sucrose, 7.5 mM KCl, 0.01% (v/v) Igepal CA-630, 10 mM DTT (Dithiothreitol), 1% (v/v) Protease inhibitors (Sigma), 0.5% (v/v) polyvinylpolypyrrolidone) at 2 ml to 1 g of fresh weight tissue was added. All subsequent steps were performed at 4 ^{o}C. Protein concentration was determined (usually 0.4 - 0.6 g total protein) with BCA assay using BSA as the standard. Homogenate was filtered through two layers of miracloth and centrifuged at 6000 g for 20 min. 20 µl of chromotek GFP or RFP trap sepharose beads (as appropriate) were added per 50 ml homogenate and incubated for 3 hours with gentle shaking. The homogenate was then centrifuged at 500 g for 5 min and the supernatant discarded. The bead slurry was washed 5 times with fresh pre-chilled extraction buffer (no polyvinylpolypyrrolidone or protease inhibitors) with 3 min incubation. The slurry was collected after the last wash and protein eluted with 2x SDS-PAGE loading buffer and taken for either LC-MS or Western blotting.

Western blotting and Immunodetection - 10% poly-acrylamide SDS-gels were run at 100/200 V and proteins electroblotted onto PVDF membranes at 250 mA (Biorad). Membranes were rinsed in TBS and blocked in 5% (w/v) non fat milk powder in TBS 0.1% tween (TBST) (w/v) for 1 hour. Primary antibodies were diluted in 0.5% (w/v) non fat milk, TBST to the following concentrations and incubated at room temperature for 1 hour. Primary antibodies were: Anti-AHA1 (H^{+}ATPase 1) 1:2 000 (Agrisera AS07 260), anti-BIP2 (luminal binding protein) 1:2 000 (Agrisera AS09 614), anti-RbcL (Rubisco Large Subunit) 1:10 000 (Agrisera AS03 037), anti-COX2 (Cytochrome Oxidase 2) 1:5 000 (AS04 053A Agrisera), anti-RFP 1:10 000 (Abcam ab34771). Membranes were washed three times in TBST before 1 hour incubation with secondary antibodies anti-rabbit-HRP (Sigma A3687) 1:10 000 or anti-hen-HRP 1:10 000 (Agrisera AS09 603). Signals were visualized using chemiluminescent substrate (Lumigen ECL, GE Healthcare) and GE healthcare Image Quant LAS 3000.

Tryptic digestion of proteins – Affinity purified proteins were separated on 4-20% Tris-Glycine nUView pre-cast gradient gels (NuSep). The SDS-PAGE gels were cut into 7 slices per affinity purification. Gel slices were washed for 30 min with 50% ACN/ 25 mM ammonium bicarbonate (ABC) at 37 ^{o}C, twice. Then 100% ACN was added for 10 min and the liquid removed. 10 mM DTT in 50 mM ABC was added to cover the gel pieces for 30 min at 56 ^{o}C shaking and the supernatant removed. 55 mM Chloroacetamide in ABC (in the dark) was applied for 20 min. The gel pieces were washed twice for 15 min with 50% ACN/ 25 mM ABC and dehydrated with 100% ACN for 10 min. 1µg of trypsin, 46 mM ABC, 5% ACN was applied at 37 ^{0}C overnight and the supernatant removed and retained. The gel pieces were washed three times by addition of 50% ACN, 5% formic acid and sonicated for 10 min and the wash supernatants were then pooled with previous supernatants. The supernatants containing the peptides were then lyophilized. The control samples, from plant expressing the fluorescent tags mCherry or GFP only were treated as above expect that the tryptic digestions were performed from the affinity beads directly, and these samples were analysed on an Orbitrap Fusion, not an Orbitrap XL as detailed below.

Mass Spectrometry - LC-MS/MS analysis was performed using a hybrid mass spectrometer LTQ-Orbitrap XL (ThermoFisher Scientific) and a nanoflow-UHPLC system (nanoAcquity, Waters Corp.) Peptides dissolved in 2% acetonitrile, 0.2% trifluoroacetic acid were applied to a reverse phase trap column (Symmetry C18, 5 µm, 180 µm x 20 mm, Waters Corp.) connected to an analytical column (BEH 130 C18, 1.7 µm, 75 µm x 250 mm, Waters Corp.) in vented configuration using nano-T coupling union. Peptides were eluted in a gradient of 3-40 % acetonitrile in 0.1 % formic (solvent B) acid over 50 min followed by gradient of 40-60 % B over 3 min at a flow rate of 250 nL min^{-1} at 40 ^{o}C. The mass spectrometer was operated in positive ion mode with nano-electrospray ion source with ID 0.02mm fussed silica emitter (New Objective). Voltage +2kV was applied via platinum wire held in PEEK T-shaped coupling union. Transfer capillary temperature was set to 200 ^{o}C, no sheath gas, and the focusing voltages in factory default setting were used. The Orbitrap, MS scan resolution of 60 000 at 400 m/z, range 300 to 2 000 m/z was used, and automatic gain control (AGC) target was set to 1 000 000 counts, and maximum inject time to 1 000 ms. In the

linear ion trap (LTQ), MS/MS spectra were triggered with data dependent acquisition method for the 5 most intense ions. The threshold for collision induced dissociation (CID) was above 1 000 counts, normal scan rate, AGC accumulation target was set to 30 000 counts, and maximum inject time to 150ms. A data dependent algorithm was used to collect as many tandem spectra as possible from all masses detected in master scan in the Orbitrap. For the latter, Orbitrap pre-scan functionality, isolation width 2 m/z and collision energy set to 35 % were used. The selected ions were then fragmented in the ion trap using CID. Dynamic exclusion was enabled allowing for 1 repeat only, with a 60 sec exclusion time, and maximal size of dynamic exclusion list 500 items. Chromatography function to trigger MS/MS event close to the peak summit was used with correlation set to 0.9, and expected peak width 7 s. Charge state screening enabled allowed only 2+ charge states and higher to be selected for MS/MS fragmentation. Control samples were analysed on an Orbitrap Fusion (ThermoScientific) with a Dionex Ultimate 3 000 nanoLC system, with a TRAP 50 cm C18 BEH column form ThermoScientific. Peptides were eluted in a gradient of 3-35 % acetonitrile in 0.1 % formic (solvent B) acid over 90 min followed by gradient of 90 % B over 5 min at a flow rate of 300 nL min^{-1} at 40 ^{o}C. The mass spectrometer was operated in positive ion mode with nano-electrospray ion source with ID 0.02mm fussed silica emitter (New Objective). Voltage +2.5 kV was applied to the metal coated tip. Transfer capillary temperature was set to 265 ^{o}C, no sheath gas, and the focusing voltages in factory default setting were used. The Orbitrap, MS scan resolution of 120 000 at 400 m/z, range 300 to 1 500 m/z was used, and automatic gain control (AGC) target was set to 400 000 counts, and maximum inject time to 50 ms. In the dual pressure ion trap MS/MS spectra were triggered for charge states 2+ to 7+ using HCD fragmentation at 35% and a maximum speed strategy where variable numbers of MS/MS spectra would be acquired within a 2 second window. The threshold for ion selection was 5 000 counts, and selected parent ions were isolated using a mass window of 1 .6 in the quadrupole. Fragment ions were detected using the a rapid ion trap scan rate, AGC accumulation target was set to 10 000 counts, and maximum inject time to 200 ms and range 100-2 000 m/z**.** Dynamic exclusion was enabled allowing for 1 repeat only, with a 45 s exclusion time, and maximal size of dynamic exclusion list 500 items.

Software processing and peptide identification - Peak lists in format of Mascot generic files were

prepared from raw data using Proteome Discoverer v1.2 (ThermoFisher Scientific). Peak picking settings were as follows: m/z range set to 300-5 000, minimum number of peaks in a spectrum was set to 1, S/N threshold for Orbitrap spectra set to 1.5, and automatic treatment of unrecognized charge states was used. Peak lists were searched on Mascot server v.2.4.1 (Matrix Science) against TAIR (version 10) database with GFP, RFP and common contaminants such as keratin added. Only tryptic peptides, were permitted with up to 2 possible miscleavages and charge states +2, +3, +4, were allowed in the search. The following modifications were included in the search: oxidized methionine (variable), carbamidomethylated cysteine (static). Data were searched with a monoisotopic precursor and fragment ions mass tolerance 10 ppm and 0.8 Da respectively. Mascot results were combined in Scaffold v. 4 (Proteome Software) and exported in Excel (Microsoft Office). Peptide identifications were accepted if they could be established at greater than 95.0% probability by the Peptide Prophet algorithm with a minimum of two unique peptides at 99% PeptideProphet probability [48].

Proteome analysis – Protein identifications and total spectrum counts were exported from Scaffold and the fold enrichment over control samples (containing fluorescent protein baits) was calculated using SaintExpress [49]. Default settings were used, where all three replicates counted equally and we did not use any known interaction information to weight interaction probabilities. Proteins were considered to be statically enriched if the SaintExpress probability score was greater or equal to 0.8 in keeping with recommendations [49, 50].

The Sungear diagram was generated in virtual plant [51]. Venn diagrams were produced in R (File S1). To identify transmembrane domains and putative signal peptides in our proteomic data we parsed a bulk download of protein data from Swiss-Prot and pTREMBL (http://web.expasy.org/docs/swiss-prot_guideline.html) using the Perl script (File S2). We also extracted protein name information and number of transmembrane domains from TAIR10 by direct download of proteins with transmembrane domains from (http://www.arabidopsis.org/ and http://www.uniprot.org). We used transmembrane domain information from TAIR10 preferentially to that from Swiss-Prot, and protein records from the manually curated Swiss-Prot preferentially to data from electronically annotated pTREMBL. Data was then amalgamated with information of

acylaton [52] and comparison to published proteomic data [33, 39, 44, 45, 53, 54] in Excel.

Constructs - N-terminal fusions of PRA1.B1 (Prenylated Rab Acceptor B1), PRA1.B2 and PRA1.F1 to RFP or YFP were generated from PCR amplification from cDNA. The amplicons were then cloned into the entry vector pENTR/D-TOPO (Invitrogen) and resulting clones confirmed by sequencing and cloned into pUB N RFP/YFP [55].

Transient expression - For microprojectile bombardment, pUB::YFP or pUB::RFP-PRA1.B1/PRA1.B2/PRA1.F1 were coated onto 1 µm gold particles and bombarded into 4- to 5-week-old leaves of pUB::RFP-RABF2b/ARA7, pUB::YFP-GOT1 and pUB::RABF1/ARA6-RFP using a Bio-Rad Biolistic PDS-1000/He particle delivery system. Bombardment sites were imaged 16 h after bombardment by confocal microscopy. Data were collected from at least two independent bombardment events and several independent plants.

Confocal Microscopy – Confocal laser scanning microscopy was performed using the laser point scanning microscope Leica SP5. YFP was excited using the 514 nm argon laser, and fluorescence emissions were captured between 520 and 550 nm for YFP. RFP was excited at 561 nm, and emission was taken between 580 and 620 nm. The sequential scan mode was used for simultaneously imaging of YFP/RFP. Images were processed using the LeicaLite and Adobe Photoshop CS4 software packages. Images are maximum projections of a consecutive series of multiple Z planes 1 µm apart.

RESULTS AND DISCUSSION

Affinity enrichment of subcellular proteomes -To identify the proteomes of various endomembrane compartments, we simplified published affinity purification methods for the enrichment of vesicle associated proteins [44, 45] by removing the sucrose density gradient step and extending the number of selected subcellular markers to create a large-scale, comparative proteomics approach. For this, we used sterile grown *A. thaliana* seedlings stably expressing GOT1, RABD2a/ARA5, RABG3f and VAMP711 fused to YFP, RABF2b/ARA7 and RABF1/ARA6 fused to the monomeric RFP,

CLC2-GFP and as controls seedlings expressing the fluorescent proteins only (Figure 1a, Table 1). All these markers were under the control of constitutive promoters ensuring sufficient expression levels and are detected in seedling leaves at their assigned subcellular localizations (Figure 1b). Total seedlings were harvested after eight days of hydroponic growth, homogenized, and the proteins extracted in buffer followed by single low speed centrifugation. Using the workflow outlined in Figure 2a, we affinity purified RFP-RABF2b/ARA7 and YFP-RABG3f from the respective transgenic lines and assessed the enrichment for possible organelle contamination using western blotting. Our approach greatly enriched the relevant bait proteins with no detectable contamination from the ER, mitochondria, chloroplasts and PM as revealed by our failure to detect BIP2 (AT5G42020), COXII (ATMG00160), RbcL (ATCG00490) and AHA1 (AT2G18960) with αBIP2, COXII, RbcL, and AHA1 antibodies, respectively (Figure 2b). No bands to either of RFP, YFP or the organelle markers were identified in affinity purifications from wild type seedlings, indicating that abundant cytosolic or membrane-associated proteins did not generally bind to the affinity beads (Figure 2b). We then proceeded to identify and compare the proteomes of seven different subcellular marker lines. We affinity purified YFP-GOT1, YFP-RABD2a/ARA5, YFP-RABG3f and YFP-VAMP711, RFP-RABF2b/ARA7, RABF1/ARA6-RFP, and CLC2-GFP from the respective transgenic *A. thaliana* seedlings using GFP and RFP beads. We refer to these affinity purifications as 'endosomal-enriched proteomes' and acknowledge that because we used liquid nitrogen to freeze plant material it is possible that larger compartments might not remain intact. In this manuscript we use the term 'compartment' as a descriptive term for any membrane-bound structure, that is not an organelle, regardless of its size.

Identification and comparison of proteins from the seven affinity enrichments - The proteins from each affinity enrichment were separated by one-dimensional gel electrophoresis and identified by mass spectrometry. The full dataset is available from the proteomics database PRIDE (accession numbers PXD001877 to PXD1885 inclusive. Table S1). All affinity enrichments contained peptides from the corresponding bait protein except for YFP-GOT1 that was only identified by peptides from YFP. This is probably because GOT1 is less amenable to tryptic digests, with only two potential peptides between 4 and 24 amino acids in length (Figure S1). Proteins were accepted as

significantly enriched by a specific bait if their SAINT Express score was 0.8 or higher [49, 50]. The SAINT scores and spectrum counts for all proteins assigned to each affinity enrichment, replicate and associated controls are summarised in Table S2. After SAINT analysis, 433 proteins were accepted being significantly enriched with at least one bait and each affinity enrichment had a number of unique and shared proteins (Table 2). Most proteins were identified from the RFP-RABF2b/ARA7 enrichment (279 proteins in total) of which 43% were uniquely assigned to this bait. The CLC2-GFP and YFP-VAMP711 baits had the smallest number of proteins assigned to their enrichments (49 and 51 proteins respectively) with smaller proportions of uniquely assigned proteins (30% and 23% respectively). RABF1/ARA6-RFP and YFP-GOT1 both had about 60 proteins assigned to their enrichments, but differed in the proportion of unique proteins assigned to each; only nine (14%) were unique to RABF1/ARA6-RFP while 30 (48%) were unique to YFP-GOT1.

As several of our bait proteins have related biological functions it is not surprising that we assigned many proteins to more than one affinity enrichment. In addition to excluding proteins that were enriched with the fluorescent protein baits only, our comparison of seven different baits provides a further measure of likely common contaminant proteins. Only five proteins were significantly enriched in all affinity purifications and four proteins were enriched with six of the seven baits. These include chaperonin 60A and 60B (CPN60; AT1G55490, AT2G28000), the ABC-type transporter PEN3/ABCG36 (AT1G59870) and two ARM repeat family proteins (AT5G53480, AT5G19820). Several chaperones and ribosomal components are considered to be contaminants in affinity purifications and we identified five Heat Shock Proteins, six additional CPN60/TCP-1 proteins and four ribosomal proteins with some, but not all, of our baits. The RFP-RABF2b/ARA7 and YFP-RABG3f enrichments contained several of these proteins and it is possible that the abundance of proteins associated with protein expression and folding are correlated with the expression level of the bait protein. BIP2 (AT5G42020), COXII (AT1G02410) RbcL (ATCG00490), that we tested for in our Western blot assessment of contamination from organelles, were not significantly enriched with any bait (Figure 2b). However, two plasma membrane ATPases were identified with specific baits and might represent contamination with plasma membrane proteins, or normal secretion, turnover and recycling of these abundant proteins. AHA1 (AT4G30190) was identified with

RFP-RABF2b/ARA7 and AHA2 (AT2G18960) with three baits.

To determine if proteins with known functions in endomembrane trafficking were identified in our affinity enrichments, we manually curated a set of endomembrane regulators from the literature (Table S3) and present the fold enrichment over controls with each of our seven baits (Table 3). This set of established endomembrane proteins was differentially enriched with each of the seven baits and largely corresponds to the known biological functions of our bait proteins.

To visualise the differences and commonalities in the seven affinity enrichments we used the Sungear tool from Virtual plant [51] (Figure 3). On the Sungear plot, the CLC2-GFP and YFP-VAMP711 enrichments appear to be distinct from the other enrichments, but, as discussed above, approximately 70% of their assigned proteomes are shared with other baits. However, there are few binary overlaps between CLC2-GFP and YFP-VAMP711, rather the shared proteins are common to many baits. The RFP-RABF2b/ARA7 and YFP-RABG3f proteomes have high numbers of uniquely assigned proteins and share a large binary overlap, possibly representing the LE/MVB compartments. There is also substantial pair wise and collective overlaps between the YFP-RABD2a/ARA5, RFP-RABF2b/ARA7 and YFP-RABG3f. The RABF1/ARA6-RFP enrichment has only nine unique proteins and is nearly a subset of RFP-RABF2b/ARA7.

Our method preferentially enriches proteins transiently associating with compartments - To compare the proteins we identified with published proteomic studies, we complied a comprehensive dataset of proteins identified in the TGN/EE [44], TGN [45], ER, Golgi, Mitochondria/Plastid, PM, Vacuole [39], Golgi [37], ER, Golgi, Mitochondria/Plastid/PM/Vacuole (combined from [33, 53]) (Table 2, Table S4). The YFP-GOT1 enrichment contained the highest proportion of previously identified proteins, likely due to the greater focus on Golgi derived proteomes in these studies. To assist with comparisons between the sets of proteins associated with each of our baits and previously published proteomes we provide Table S4.

To identify possible cargo proteins from transient interactions of coat proteins and trafficking

regulators, we extended our bioinformatic analysis to include predicted transmembrane domains and membrane associated modifications (Table S4). In the 433 proteins enriched by at least one of our affinity baits, we identified 64 proteins with one transmembrane domain and 58 with multiple transmembrane domains, 12 RAB GTPases with membrane association lipid modifications, and a diverse set of 63 potentially S-acylated proteins [52] (Table S4). The remaining 66% of our dataset (285 proteins) did not have any membrane association motifs and are putative cargo proteins. The proportions of proteins in each proteome with membrane association motifs varied across the enrichments; YFP-GOT1 had the greatest at 77%, whilst CLC2-GFP and YFP-RABG3f the smallest proportion of membrane-associated proteins, 24% and 22%, respectively (Table 2, Figure S2). The proportion of YFP-GOT1 membrane associated proteins is remarkably similar to Golgi or TGN proteomic studies such as Drakakaki et al. [44] (77%) or Parsons et al. [37] (75%). That our other affinity baits, such as RFP-RABF2b/ARA7, show lower proportions of membrane associated proteins is likely to be a consequence of our choice of bait protein for affinity purification and the biological functions of these small GTPases. By targeting RAB regulators of vesicle trafficking we identify more proteins with transient membrane interactions, such as vesicle coats (e.g. COPI and COPII), motor proteins (e.g. myosins), and endomembrane tethers than previous proteomic studies (Table 3, Table S4).

Affinity Enrichment of CLC2-GFP identifies Clathrin Mediated Endocytosis components - We included CLC2-GFP in this comparative proteomics study because we expected that the proteins associated with CLC2 would be substantially different to those enriched with RAB GTPase baits. Our CLC-GFP enrichment recovered established clathrin interacting proteins and provides the first independent supportive evidence for the recently defined association of TPLATE complex components with clathrin (Table 3). As expected, some of the most abundant proteins in the CLC2-GFP enrichments were; CHC1 (Clathrin Heavy Chain 1, AT3G11130), CHC2 (AT3G08530), CLC1 (AT2G20760) and, the bait, CLC2 (AT2G40060). Several accessory proteins involved in CME were also assigned to the CLC2-GFP enrichment, including components from the TPLATE complex [47, 56] (Table 3). Specifically; two SH3 (SRC homology) domain containing proteins (SH3PH, AT4G34660 and TASH3, AT2G07360), an ENTH (Epsin N Terminal Homology), domain containing protein ECA4 (AT2G25430),

a WD40 protein, TWD-40-2 (AT5G24710) and a calcium binding EF hand protein EH2 (AT1G21630).

The TPLATE complex appears to persist at the plasma membrane after removal of AP2 and possibly through scission [56]. We identified TPLATE components and dynamin (DRP3a, AT4G33650) in our CLC2-GFP enrichments, (but not AP2 or TPLATE itself), possibly capturing several different stages of interactions. Interestingly we identified AP2, TPLATE, CAP1 and other members of this complex with RFP-RABF2b/ARA7 and YFP-RABD2a/ARA5 (Table 3). Scission of vesicles from membranes is mediated by large GTPases such as dynamins and we note that several dynamins were abundantly represented in many of our enrichments including controls and therefore did not pass our significance threshold. Only DRP3a (AT4G33650) passed significance thresholds with three baits, DRP3b (AT2G14120) with RFP-RABF2b/ARA7 and DRP2a (AT1G10290) with YFP-VAMP711.

One of the less well-characterised proteins assigned to our CLC2-GFP enrichments was synaptotagmin A (SYTA, AT2G20990) that regulates endocytosis and plasmodesmatal viral movement [57]. Lewis et al. [57] demonstrated the co-localisation of SYTA with the endocytic tracer dye FM4-64 and with RABF1/ARA6-GFP. We identified SYTA with CLC2-GFP, YFP-RABD2b/ARA5 and RFP-RABF2b/ARA7 baits. *A. thaliana* has five homologues of synaptotagmin and little is known about the functions of this family. STY1 is the best characterized and functions in resealing plasma membrane after stress [58, 59] and SYT2 is required for unconventional secretion, namely ER/Golgi independent pathways and does not co-localize with FM4-64, RABF1/ARA6 or other endosomal markers, but only with the Golgi apparatus [60].

The YFP-GOT1 enrichment is consistent with previously published Golgi proteomes – In all eukaryotes, the Golgi is an essential organelle for protein secretion, *N*-linked protein glycosylation and, in plants, is essential for the biogenesis and secretion of cell wall polysaccharides. YFP-GOT1 is a marker for the Golgi and is homolog of yeast GOT1p that localised to *cis*-Golgi [26, 61]. Little is known about the precise location and function of GOT1 within the Golgi of *A. thaliana*. However, as several Golgi proteomic studies have been performed, we included YFP-GOT1 as an additional comparison for the RAB GTPase baits and expected it to be an outlier. We compared our YFP-GOT1

enrichment with published proteomic analyses of Golgi compartments (Figure 4a, Table S4) [33, 37, 39, 53]. Two thirds (41/62) of proteins identified with our YFP-GOT1 bait were previously identified in Golgi proteomic analyses (Figure 4a).

In common with previously published Golgi proteomes, Golgi-localised enzymes were well represented in our YFP-GOT1 enrichment including; galacturonosyltransferases (GAUT, AT2G38650, AT3G02350, AT3G25140, AT3G61130), xylose synthase (AT2G47650) and xylose transferase (KKT5, AT1G74380) and a UDP-D-glucuronate 4-epimerase 6 (GAE1, AT3G23820). The GAUT (galacturonosyltransferase) proteins all have one TM domain are believed to be localised to discrete sub-compartments within the Golgi, additionally GAUT1 is known to interact with GAUT7 [62]. We identified nine S-adenosyl-L-methionine-dependent methyltransferases (SAM; AT1G29790, AT1G78240, AT4G00750, AT4G14360, AT5G64030, AT4G18030, AT1G26850, AT1G29470, AT3G23300). Nikolovski et al. [39] suggest that the SAM enzymes are involved in methylation of glycans. Four Endomembrane Family Protein 70 (EMP70; AT4G12650, AT5G35160, AT5G25100, AT1G14670) were also identified in our YFP-GOT1 dataset. EMP70 proteins contain nine transmembrane domains and their function is not known. Our YFP-GOT1 enrichment also contained the cis-Golgi-localised glycosyltransferase, alpha-mannosidase II (GMII, AT5G14950) that was not identified in previous Golgi proteomes. This protein is frequently used as a marker of the Golgi by western blotting (eg. [63]).

Although most of the proteins assigned to our YFP-GOT1 enrichment have been identified in published Golgi studies, our YFP-GOT1 enrichment contained fewer proteins (62 proteins) than were obtained by Parson et al. [37] (371 proteins), Nikoloviski et al. [39](197 proteins) or Sadowski et al. [53] (91 proteins). Our YFP-GOT1 enrichment shows an overlap of only seven proteins with the 146 proteins identified in the CFP-SYP61 TGN/EE proteome [44] and just four proteins with the TGN proteome [45] (Figure 4a, Table S4). These differences suggest that YFP-GOT1 labels and enriches for a distinct area of the Golgi, possibly on the *cis*-Golgi side. To further test the specificity of our method, we compared the YFP-GOT1 enrichment to the YFP-VAMP711 enrichments (Figure S3). These two proteomes should be distinct due to their spatial and functional differentiation to

the Golgi and Vacuole respectively, and the data supports this statement (as the H_0 of random assignment of proteins can be rejected with a $X^2 - 43.3$, $p<0.001$).

As expected, the YFP-GOT1 enrichment differed from the proteins identified with our RAB baits and most obviously in that few of the known endomembrane regulatory proteins were identified with this bait (Table 3). However, two RAB regulatory proteins (PRA1.B2, PRA1.B4) and a Qb-SNARE and a dissociation factor, (GOS12 and NSF) were identified with YFP-GOT1 and were also seen in previous studies. Additionally, the R-SNARE (VAMP722/SAR1) was identified with YFP-GOT1 (and four other of our baits) but was not identified in previous proteomic studies of the Golgi and TGN. Both of these SNARE proteins are known to localise to the Golgi and, in yeast, GOT1 facilitates SNARE fusion [64, 65] (Table 3, Table S4).

Plants differ from animals in that they possess numerous highly mobile Golgi bodies that are tightly associated with the ER [66-69]. COP coated vesicles mediate traffic between the ER and the cis-Golgi and might be expected to be enriched with our GOT1 bait. However, the COP proteins we identified assigned to the small GTPase baits (RABD2a/ARA5 and RABF2b/ARA7). The structural roles of the proteins we selected as baits might explain this apparent discrepancy; GOT1 has four TM domains while the RAB GTPases associate transiently with membranes. In yeast GOT1 is packaged into COPII vesicles and cycles between ER and Golgi [70]. It is possible that the YFP-tag interfered with GOT1 role in COPII vesicles, or that the tag was masked from the affinity matrix by coatmers. In yeast GOT1 has a role in membrane fusion and we identified a novel fusigenic protein with YFP-GOT1; the large, dynamin-like, GTPase RHD3 (AT3G13870). RHD3 has homology to atlastins and is proposed to mediate ER tubule formation and fusion [60, 68, 71].

ARA5 and ARA7 RAB GTPase mediate trafficking associated with the TGN - RAB GTPases cycle on and off membranes and recruit proteins (known as RAB effectors) such as coat proteins and tethers, to establish a unique, but transient, membrane identity. Our objective in selecting several RAB GTPases as baits for affinity purification was to capture some of the transient trafficking events associated with the TGN and not to characterise a steady-state composition, which has been

established by Groen et al. 2014 [45]. Of all our baits, the YFP-RABD2a/ARA5 and RFP-RABF2b/ARA7 enrichments showed the greatest similarity to the TGN proteomes identified using CFP-SYP61 and VHA-a1-GFP affinity purifications by [44, 45] (Figure 4b, Table S4).

YFP-RABD2a/ARA5 is localised throughout the TGN and EE in punctate structures that are sensitive to BFA and is (partially) labelled with FM4-64 and co-localises with VHA-a1 [26, 28]. Of the 120 proteins we assigned to the YFP-RABD2a/ARA5 enrichments, 23 proteins were common to CFP-SYP61 and 17 to VHA-a1-GFP enrichments (Figure 4b). We did not identify VHA-a1 in the YFP-RABD2a/ARA5 enrichment but did identify another VHA-A ATPase (AT1G78900); this protein was also identified in CFP-SYP61 affinity purifications [44]. Surprisingly, there was substantial overlap between our YFP-RABD2a/ARA5 and RFP-RABF2b/ARA7 enrichments (85 proteins). The RFP-RABF2b/ARA7 enrichment did not contain RABD2a, but did contain RABD2b possibly supporting separate roles for these two RABD2 proteins as reported by [28].

Our RFP-RABF2b/ARA7 enrichments showed an overlap of 20/105 proteins that were identified in VHA-a1-GFP affinity enrichments by Groen et al. [45], including VHA-a1 (AT2G28520). VHA-a-1 was also identified in CFP-SYP61 enrichments by Drakakaki et al. [44] and our RFP-RABF2b/ARA7 enrichment had an overlap of 35/146 with the CFP-SYP61 proteome [44]. That we did not identify SYP61 in any of our seven affinity enrichments that could suggest distinctness of the proteomes associated with each bait. The 279 RFP-RABF2b/ARA7 associated proteins were the most numerous and diverse of any of our enrichments, suggesting that RABF2b/ARA7 might have a general role in EE/TGN and LE/MVB trafficking.

RAB GTPase regulator proteins exhibit varied associations - We identified many proteins that regulate RAB GTPase function in YFP-RABD2a/ARA5 and RFP-RABF2b/ARA7 enrichments (Table 3). All RAB GTPases have a hydrophobic membrane association modification, typically prenylation, and as membrane association is a pre-requisite for RAB GTPase activation by exchange of GDP for GTP, proteins that bind to this motif prevent the RAB from associating with the membrane. Therefore, these regulatory proteins are known as GDP dissociation inhibitors (GDIs; reviewed [72-74]). We

identified GDI1 with RFP-RABF2b/ARA7 enrichments. For a RAB GTPase to associate with a membrane the GDI must be removed by the action of a GDI dissociation Factor (GDF) [70, 75-77]. The homologs of yeast GDFs in *A. thaliana* are the Ypt Interacting protein (YIP) and Prenylated Rab GTPase acceptor 1 (PRA1) family [78, 79]. We identified YIP4b with both RFP-RABF2b/ARA7 and YFP-RABD2a/ARA5 baits and PRA1.B4 and PRA1.B1 with RFP-RABF2b/ARA7 and YIP5b and YFP-RABD2a/ARA5. GDI1 was not identified in either CFP-SYP61 or VHA-a1-GFP proteomes, as expected for a protein that associated with the cytoplasmic complex of a RAB. The PRA1 and YIP proteins were identified in previously characterised TGN proteomes.

RABs are poor GTPases and their activity at membrane surfaces is controlled by guanine nucleotide exchange factors (GEFs) and GTPase-activating proteins (GAPs) that facilitate nucleotide exchange and hydrolysis. We identified VPS9a, the activating GEF for RABF GTPases [80] in RFP-RABF2b/ARA7 (and YFP-RABG3f) enrichments. The interaction of RABF2a/ARA7 with VPS9a is well characterised: In yeast-two hybrid assays and by co-immunoprecipitation, VPS9a physically interacts with RABF proteins [80] and the structure of the RABF2b/ARA7 - VPS9a complex has been solved [81]. In the YFP-RABD2a/ARA5 enriched proteins we identified one core TRAPPI component, TRS85 (AT5G16280) [82]. There are two distinct TRAPP complexes an animal cells; TRAPPI and TRAPPII and in yeast the TRAPPI complex is GEF to the RAB1/RABD homologue, Ypt1p [83, 84]. Due to their co-enrichment we suggest that the TRAPPI may also be a GEF for RABD2a/ARA5. TRAPP components were identified in CFP-SYP61 and VHA-a1-GFP proteomes, but VPS9a was not.

We identified four other GEFs, of the ADP ribosylation factor (ARF) family, in both YFP-RABD1a/ARA5 and RFP-RABF2b/ARA7 enrichments, specifically; GNOM (AT1G13980), BIG5/MIN7 (AT3G43300), BIG2 (AT3G60860) and BIG3 (AT1G01960). These four GEFs can be all be inhibited by BFA and are associated with coated vesicle formation [1]. The best-known plant ARF-GEF is GNOM due to its regulation of auxin transporter (PIN) recycling and partial co-localisation with FM4-64 and [85]. GNOM has recently been shown to be primarily localised to the Golgi and to regulate TGN structure [86]. BFA treatment seems to pull the Golgi into a body that is surrounded by TGN and this is consistent with recent work disambiguating TGN structure [87]

and suggests that GNOM influence on ARA7 localisation is indirect [85, 86]. Less is known about the functions of BIG1 to BIG4, and BIG5/MIN7 has a role in plant immunity and PIN trafficking at the TGN/EE [88, 89]. Of these four ARF-GEFs, only BIG5/MIN7 was previously identified in the VHA-a1-GFP affinity purification.

Identification of candidate novel GARP components - RAB GTPases regulate compartment identity by recruiting intra-membrane tethers and SNARE proteins, which promote the direct fusion of membranes once tethering factors have brought them into close contact, reviewed [87]. The combinations of RAB, tethers and SNARE proteins dictate the interactions a compartment can make and are further regulated by SNARE attachment proteins (SNAP) and Sec1/Munc18 (SM) proteins.

We identified tether proteins and complexes with RFP-RABF2b/ARA7 and YFP-RABD2a/ARA5 baits including; Conserved Oligomeric Golgi (COG), Golgi-associated retrograde protein (GARP) and the Golgin GC6 (with RFP-RABF2b/ARA7 only). The COG and GARP are both multi-subunit complexes of the CATCHR group of long tethers and are known to be effectors of RAB GTPases [90]. These complexes were not identified in previous proteomic analyses of the TGN.

In yeast the COG complex coordinates retrograde Golgi traffic and interacts with RAB, SNARE and golgin proteins (reviewed in [88, 91, 92]). In plants, homologues of the COG complex have been predicted on the basis of sequence similarly [93] but there is little experimental evidence for the function and location of this complex in *A. thaliana*. We identified five of the eight putative COG subunits with RFP-RABF2b/ARA7 (COG1, COG2, COG5, COG7, COG8) and two with YFP-RABD2a/ARA5 enrichments (COG 7, COG8; Table 3). The *A. thaliana* COG2 (AT4G24840) subunit has recently been shown to recruit the exocyst subunit, EXO70A1, to punctate structures at cortical microtubules during xylem formation [94]. The putative COG1 subunit (AT5G16300) has also been annotated as a possible GARP complex subunit (VPS51) [95]. We have placed AT5G16300 in the COG group of Table 3 but it could equally belong to the GARP complex.

The primary role of the tetrameric GARP complex is in the reception of endosome derived vesicles

at the TGN (recently reviewed in [90]). GARP is a highly conserved complex consisting of VPS51, VPS52, VPS53, VPS54 subunits [96]). In yeast the VPS52 subunit interacts with GTP bound RAB, Ypt6 [97, 98]; RABH is the homologous group in *A. thaliana*). We identify the VPS52 subunit with five of our affinity enrichments (RABF1/ARA6-RFP, YFP-RABG3f and CLC2-GFP baits in addition to YFP-RABD2a/ARA5 and RFP-RABF2b/ARA7), whereas the other three GARP components appear to have a more limited distribution (Table 3). We suggest that VPS52 might be the GTPase interacting component of the GARP complex in plants. VPS51 (Unhinged; At4g02030) has recently been shown to interact directly with VPS52 and to co-localise with RABF2a and SYP61 [95]. *A. thaliana* contains three putative VPS51 homologues and the identification of VPS51 with the other three GARP subunits in our RFP-RABF2a/ARA7 enrichments further supports Pahari et al. Notably VPS51 is not significantly enriched with YFP-RABD2a/ARA5 but another putative VPS51/COG1 homologue (AT5G16300) is.

The transition from the *TGN/EE to LE/MVB* – To the best of our knowledge there are no comparable published proteomes to our YFP-RABG3f, YFP-VAMP711 and RABF1/ARA6-RFP enrichments. Analysis of these proteomes should help elucidate the biological mechanisms of transition from RAB5 labelled LE/MVBs to RAB7 labelled LE/MVBs. Furthermore, 167 proteins of our RFP-RABF2b/ARA7 enrichments did not overlap with TGN associated proteomes (Figure 4b). These four baits have many proteins in common and represent the late endosomes to the LE/MVB and vacuole (Figure 5b).

One striking observation is that the proteomes of RFP-RABF2b/ARA7 and RABF1/ARA6-RFP are very, but not significantly (X^2 – 0.6, $p>0.1$), similar with an overlap of 49 proteins (Figure 5a). The RABF1/ARA6-RFP proteome is much smaller than RFP-RABF2b/ARA7, with just 14 proteins not seen in RFP-RABF2b/ARA7 and only eight proteins are unique to RABF1/ARA6-RFP (compared to all our baits). These eight proteins are; TRS120, two ARM repeat family proteins (AT1G65220, AT3G05040) two chloroplast associated proteins (LHC, AT3G08940; TOC, AT3G46740) and three enzymes (PFK, AT4G29220; KCS19, AT5G04530; GPTA4, AT1G01610). The RABF regulator VPS9a (AT3G19770) was identified with both baits but only passed our significance test with RFP -RABF2b/ARA7 [80]. An

important difference between the RFP-RABF2b/ARA7 and RABF1/ARA6-RFP endosomes is the presence of numerous TGN/EE localised proteins, (RABA1b, SYP43, VHA-a1, SCAMP1) and many long tether complexes in the RFP-RABF2b/ARA7 but not RABF1/ARA6-RFP enrichments [8, 72, 99].

That the proteins identified with RABF1/ARA6-RFP are nearly a subset of RFP-RABF2b/ARA7 is in accordance with observations that RABF2b endosome populations mature into the RABF1/ARA6 endosomes [25, 29, 46, 100]. Despite the similarities in their associated proteins, RABF1/ARA6 and RABF2b/ARA7 labelled LE/MVBs are distinct. RABF2b/ARA7 LE/MVBs are more susceptible to application of the drug BFA than RABF1/ARA6 LE/MVBs [25]. Furthermore, RABF2b/ARA7 co-localises to a greater extent with the SNARE VAMP727 whilst RABF1/ARA6 co-localises to a greater extent with the SNAREs SYP21 and SYP22 [25]. The BFA-sensitive ARF-GEFs and VAMP727 were significantly enriched with RFP-RABF2b/ARA7 but not with RABF1/ARA6-RFP. An alternative explanation for some of the differences between our RFP-RABFb/ARA7 and RABF1/ARA6-RFP enrichments might be due to the expression level of these constructs, or in their relative efficiency in binding to the affinity matrix.

The YFP-RABG3F enrichment contained 136 proteins and has an overlap of 78 proteins with RFP-RABF2b/ARA7 identified proteins and the RABF1/ARA6-RFP and YFP-VAMP711 baits share 34 and 25 proteins respectively with this enrichment (Figure 5b)**.** The greatest diversity of RAB proteins were identified with RABG3f enrichments but only two of the five ARF GEFs were identified in total (Table 3 and Table S4). Other RAB proteins identified with YFP-RABG3f included members of the RABA, RABC, RABD, RABF and RABH families. The RAB dissociation inhibitors GDI1 and GDI2 were abundantly represented with the YFP-RABG3f bait (assessed by fold enrichment over control samples), and YFP-RABG3f was the only bait to enrich for GDI2. Forty-one other proteins were specific to the YFP-RABG3f proteome (compared to all seven baits).

The LE/MVB is characterised by luminal vesicles that are formed by the ESCRT complex. We did not identify any core ESCRT components with YFP-RABG3f enrichments, but the ATPase that promotes the disassembly of the ESCRT complex, VPS4, was identified with RFP-RABF2b/ARA7 and

YFP-RABG3f baits. Two components of the retromer complex were identified only with the YFP-RABG3f enrichments (VPS35A, VPS35B). The retromer recycles vacuolar sorting receptors back to the Golgi/TGN. Zelaznya et al 2013 demonstrated that VPS35 interacts with RABG3f. The retromer consist of two subcomplexes; VPS35-VPS26-VPS29 and a sortin nexin (SNX) (reviewed [101]). We did not identify VPS26 or VPS29 in any enrichment but we did identify SNX1 with RFP-RABF2b/ARA7, with which is known to co-localise with them [102]. In addition, two cargo recognition Vacuolar Sorting Receptors, (VSR1 and VSR4) were identified with RFP-RABF2b/ARA7 enrichment only and VSR3 was identified with YFP-VAMP711 baits. These VSRs are involved with sorting cargo from the TGN to the lytic vacuole and to the seed protein storage vacuole [103].

SNAREs at the vacuole - Our YFP-VAMP711 enrichment contains 51 proteins, of which 12 are unique to VAMP711. Interestingly we identified only two SNARE proteins in the YFP-VAMP711 enrichment, VAMP721 and SYP51. These three proteins cannot form a partial SNARE complex, as both VAMP711 and VAMP721 are both R-SNAREs [72]. SYP51 is, however, a known interaction partner of VAMP711 and may function as an 'interfering' SNARE [104]. Of particular interest is the identification of SEC12 in the YFP-VAMP711 proteome, this GEF has been implicated in regulating the exit of vacuolar proteins from the ER. It may be that the vacuole represents another localisation for SEC12 [105].

Microscopic analysis of Golgi and TGN/EE co-purifying proteins - To verify that co-purifying proteins are present at the compartments affinity purified with our baits, we monitored the subcellular localization of some of the identified proteins using confocal microscopy. We focused on the YFP-GOT1 and RFP-RABF2b/ARA7 proteomes and selected PRA1 proteins for further study as they are believed to regulate RAB GTPase function through removing the inhibitory GDI [78] although direct evidence is lacking in plants [106]. In *A. thaliana*, the PRA1 family has expanded to include 19 members and their subcellular localizations are thought be varied [78, 107]. We identified PRA1.B1 (AT3G56110) in both the YFP-GOT1 Golgi and RFP-RABF2b/ARA7 proteomes, whereas PRA1.B2 (AT2G40380) was found in the YFP-GOT1 proteome only (Table 3). By contrast, no spectrum counts matching peptides for PRA1.F1 (AT1G17700) were identified in any of our proteomes (Table S4),

although it belongs to the same protein family as PRA1.B1 and PRA1.B2 [78]. These observations suggest distinct localization patterns among the PRA1 proteins of the same family.

Using live-cell imaging, we observed partial co-localization of YFP-/RFP-tagged PRA1.B1 with both RFP-RABF2b/ARA7 and YFP-GOT1, respectively (Figure 6, Figure S4). Additionally, YFP/RFP-PRA1.B1 was present at some compartments that were not positive for YFP-GOT1 or RFP-RABF2b/ARA7, showing PRA1.B1 has a wider subcellular localisation to both Golgi and endosomes. RFP-PRA1.B2 was predominantly co-localized with YFP-GOT1 (Figure 6, Figure S4), in agreement with predictions from our proteomic analysis. YFP/RFP-PRA1.F1 was found to be closely associated with YFP-GOT1, RFP-RABF2b/ARA7, and RABF1/ARA6-RFP-positive compartments while not exhibiting strong co-localization (Figure 6, Figure S4), which is consistent that this PRA1 protein was not identified in our endomembrane proteomes. All these observations corroborate the findings from our proteome analysis and demonstrate that our data can be used to predict the localization of a protein within the cell. In contradiction to our results that RABF2b/ARA7 and PRA1.F1 do not co-localise, a previous study reports co-localisation between mRFP-ARA7 and PRA1.F1-GFP, this likely to be due to the differences in the systems used for expression [78].

CONCLUSIONS

Recent reviews and discussions [13, 54, 108] have highlighted the need to identify additional regulatory proteins and cargo of endosomal and secretory pathways. To meet this need, Tables 3 and S4 provides a potential resource for the community to explore the proteomes we present here in the context of published datasets. Our results demonstrate the utility of affinity purification for the exploration of endomembrane proteomes, and our method is comparable in efficacy to other endomembrane enrichment protocols. Furthermore our proteomic data yields unprecedented biologically significant resolution between separate endocytic compartments (for example the common and specific proteins in Figure 5b) and can be used to predict the localisation of proteins as evidenced by the PRA1 family localisations (Figure 6).

It is important to note that endosomes and membrane fractions associated with a specific marker

protein exist as heterogeneous populations within the cell and that affinity purification will pool these populations. For example, we identify GDI1 and GDI2 that only associate with the non-membrane fraction of prenylated RABs, and we identify PRA1 family proteins with YFP-RABG3f and RFP-RABF2b/ARA7. PRA1 proteins are GDFs involved in the positive regulation of RAB GTPases by removing the inhibitory GDIs. We demonstrate differential localisation of three related PRA1 GDFs and find there were no recognisable GDFs in the RABF1/ARA6-RFP proteome and that neither PRA1.B1, B2or F1 co-localise with ARA6 (Figure 6) when analysed with confocal microscopy. As ARA6 has a different lipid association modification (n-myristolation) [29] to all other RABs (prenylation) [72] and the known GDF machinery is believed to regulate RABs through interaction with the prenylation modification [78], we hypothesise that ARA6 has a set of as yet unidentified non-canonical GDF machinery. Furthermore we identify known cargoes of endosomes such as PIN3 [109], PEN3 [110], CESA [111] (Table S4). We emphasise that our data is a starting point for further work and not an end point in itself; additional validation from the community is required to properly establish the proteomes we have pioneered.

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Table 1. Affinity purification baits and their homologs in mammals and yeasts. ATG numbers and *A. thaliana* short names are from TAIR (http://www.arabidopsis.org/). Homologs were identified from published works [24, 26, 47, 112]. Abbreviations: EE, early endosomes, LE, late endosomes, TGN trans-Golgi Network, SV Secretory Vesicles, Ton Tonoplast, MVB Multi-Vesicular Body.

Table 2. Numbers of proteins identified in affinity purifications. Proteins that were identified in and unique to (according to criteria in materials and methods) each affinity purification. Percentage of proteins identified in selected previous proteomic studies was calculated from whether a protein was identified in Nikolovski et al. 2012, Parsons et al. 2012, Sadowski et al. 2008, Dunkley et al. 2006, Drakakaki et al. 2012. Percentage of proteins with membrane associations was calculated using annotations from Uniprot (http://www.uniprot.org) and TAIR (http://www.arabidopsis.org/) as described in the materials and methods.

Table 3. Endomembrane regulators identified in affinity purifications. Proteins significantly enriched with each affinity purification are marked with a grey background and the fold enrichment over that of control baits is shown. Significant and fold enrichment was calculated using SAINT Express. NS; Proteins that were identified in an enrichment, but not significantly. A: No peptides to this protein were identified. Annotations for inclusion in a complex were manually curated from literature [26, 47, 56, 65, 78, 79, 82, 93, 101, 113-118].

Table 1.

ATG number	*A.thaliana*	Mammals	*S.cerevisae*	*S.pombe*	Expected locations	Construct
AT3G18820	RABG3f	RAB7	YPT7	YPT7	LE/MVB/Ton	YFP-RabG3f
AT4G19640	ARA7/RABF2b	RAB5	YPT51/52/53	YPT5	LE/MVB	RFP-RABF2b/ARA7
AT3G54840	ARA6/RABF1	RAB22			LE/MVB	RABF1/ARA6-RFP
AT1G02130	ARA5/RABD2a	RAB1	YPT1	YPT1	Golgi/TGN/EE/SV	YFP-RABD2a/ARA5
AT4G32150	VAMP711	VAMP7	YKT6	sec22	Ton	YFP-VAMP711
AT2G40060	CLC2	CLTB	CLC1	CLC	CCV	CLC2-GFP
AT3G03180	GOT1	GOT1A	GOT1p	GOT1	Golgi	YFP-GOT1

Table 2.

	Total number of proteins	Number of unique proteins	% previously identified	% membrane associated
YFP-RABD2a/ARA5	120	22	18%	34%
RABF1/ARA6-RFP	63	9	14%	33%
RFP-RABF2b/ARA7	279	121	43%	33%
CLC2-GFP	49	15	31%	24%
YFP-GOT1	62	30	48%	77%
YFP-RABG3f	136	42	31%	22%
YFP-VAMP711	51	12	24%	41%

Table 3.

ATG number	Group	Short name	YFP-RABD2a/ ARA5	RABF1/ ARA6-RFP	RFP-RABF2b/ ARA7	CLC2-GFP	YFP-GOT1	YFP-RABG3f	YFP-VAMP711
AT5G22780	CCV	Adaptin α2	NS	NS	11	NS	NS	A	A
AT2G25430		ECA4	NS	NS	NS	73	A	43	NS
AT4G32285		CAP1	NS	NS	60	NS	A	A	A
AT3G11130		CHC1	NS	NS	NS	29	NS	NS	NS
AT3G08530		CHC2	NS	NS	NS	60	NS	A	A
AT2G20760		CLC1	A	NS	NS	18	A	A	A
AT2G40060		CLC2	NS	NS	A	110	NS	A	A
AT4G33650		DRP3A	8	NS	10	7	NS	NS	NS
AT1G10290		DRP2A	NS	NS	NS	NS	NS	NS	3
AT4G34660		SH3PH	A	A	NS	40	A	A	A
AT1G52360	Coatomer	SEC27p	5	4	10	NS	NS	7	NS
AT4G31480		SEC26p	NS	A	A	NS	A	11	NS
AT4G31490		SEC26p	NS	NS	12	NS	NS	A	A
AT1G62020		RET1p	16	16	21	NS	NS	12	7
AT2G21390		RET1p	6	7	9	NS	NS	6	NS
AT5G05010		RET2p	10	NS	10	NS	NS	NS	NS
AT3G63460		SEC31B	8	6	10	NS	NS	NS	NS
AT5G16300	COG	COG1/VPS51	33	NS	120	NS	NS	NS	NS
AT4G24840		COG2	NS	NS	37	NS	NS	NS	A
AT1G67930		COG5	NS	NS	20	A	NS	A	A
AT5G51430		COG7	27	NS	50	NS	A	NS	NS
AT5G11980		COG8	30	NS	47	NS	NS	NS	A
AT2G27600	ESCRT	VPS4	NS	NS	7	A	NS	8	A
AT5G03540	EXOCYST	EXO70A1	NS	NS	60	NS	A	NS	A
AT5G59730		EXO70H7	NS	NS	30	NS	A	A	A
AT5G49830		EXO84B/VPS51	NS	NS	40	NS	NS	23	NS

AT5G12370		SEC10	9	NS	12	NS	NS	NS	NS
AT1G47550		SEC3A	NS	NS	NS	NS	NS	33	NS
AT1G76850		SEC5A	NS	NS	107	NS	NS	NS	NS
AT3G10380		SEC8	20	12	29	10	NS	12	NS
AT4G02030	GARP	VPS51	NS	NS	8	NS	A	NS	A
AT1G71270	GARP	VPS52	33	40	50	40	NS	37	NS
AT1G50500	GARP	VPS53	37	NS	87	NS	NS	60	NS
AT4G19490	GARP	VPS54	15	NS	46	NS	NS	NS	NS
AT3G60860	GEF	BIG2	24	NS	60	NS	A	NS	NS
AT1G01960	GEF	BIG3	15	NS	33	8	NS	9	NS
AT3G43300	GEF	BIG5/MIN7	16	NS	23	NS	NS	11	NS
AT1G13980	GEF	GNOM	53	NS	97	NS	NS	NS	A
AT2G01470	GEF	SEC12	NS	NS	NS	A	NS	NS	33
AT1G16920	GTPase	AtRABA1b	7	NS	5	NS	NS	NS	NS
AT4G18800	GTPase	AtRABA1d	NS	NS	33	NS	NS	NS	116
AT3G46830	GTPase	AtRABA2c	14	NS	NS	A	NS	38	NS
AT4G17170	GTPase	AtRABB1c	NS	NS	NS	NS	NS	6	4
AT1G43890	GTPase	AtRABC1	NS	12	NS	NS	NS	6	A
AT3G11730	GTPase	AtRABD1	A	NS	NS	NS	NS	30	NS
AT1G02130	GTPase	AtRABD2a	56	NS	NS	NS	NS	NS	NS
AT5G47200	GTPase	AtRABD2b	NS	NS	10	NS	NS	11	7
AT3G54840	GTPase	AtRABF1	NS	195	NS	NS	A	NS	A
AT4G19640	GTPase	AtRABF2b	100	272	2330	NS	NS	76	NS
AT3G18820	GTPase	AtRABG3f	NS	NS	NS	A	NS	120	9
AT2G44610	GTPase	AtRABH1b	NS	NS	NS	NS	NS	6	NS
AT3G56110	RAB regulator	AtPRA1.B1	NS	A	117	A	53	NS	A
AT2G40380	RAB regulator	AtPRA1.B2	NS	A	NS	A	60	NS	A
AT2G38360	RAB regulator	AtPRA1.B4	NS	NS	110	A	NS	NS	NS
AT2G44100	RAB regulator	GDI1	NS	A	57	A	A	657	A
AT3G59920	RAB regulator	GDI2	NS	A	NS	A	A	177	A

AT3G19770		VPS9a	A	NS	130	A	A	A	A
AT4G30260		YIP4b	62	NS	104	A	A	NS	NS
AT3G05280		YIP5b	80	A	NS	A	NS	NS	60
AT1G75850	RETROMER	VPS35A	NS	A	NS	A	A	40	A
AT2G17790	RETROMER	VPS35B	NS	NS	NS	A	A	43	A
AT5G06140	RETROMER	SNX1	NS	NS	53	NS	A	NS	NS
AT2G45200	SNARE	GOS12	NS	A	A	NS	40	A	A
AT4G04910	SNARE	NSF	70	NS	113	NS	70	NS	93
AT3G05710	SNARE	SYP43	NS	A	23	A	A	A	NS
AT1G16240	SNARE	SYP51	NS	NS	NS	A	A	NS	53
AT4G32150	SNARE	VAMP711	A	NS	NS	A	NS	A	1713
AT1G04750	SNARE	VAMP721	A	A	A	A	A	A	167
AT2G33120	SNARE	VAMP722	33	9	16	NS	6	18	A
AT3G54300	SNARE	VAMP727	NS	NS	40	A	A	A	A
AT3G27530	Tether	GC6	NS	NS	17	NS	NS	NS	A
AT1G21630	TPLATE complex	EH2	NS	NS	NS	20	A	A	A
AT2G07360	TPLATE complex	TASH3	8	NS	6	5	A	A	NS
AT5G57460	TPLATE complex	TML	23	NS	NS	NS	A	A	A
AT3G01780	TPLATE complex	TPLATE	NS	7	10	NS	NS	NS	NS
AT3G50590	TPLATE complex	TWD40-1	6	NS	8	NS	NS	A	A
AT5G24710	TPLATE complex	TWD40-2	NS	NS	NS	10	A	NS	A
AT5G11040	TRAPP	TRS120	NS	23	NS	A	A	NS	NS
AT5G16280	TRAPP	TRS85	417	NS	NS	A	NS	53	NS
AT3G52850	VSR	VSR1	NS	NS	52	A	NS	A	NS
AT2G14740	VSR	VSR3	A	A	A	A	A	A	47
AT2G14720	VSR	VSR4	NS	NS	147	A	A	NS	A
AT2G20990		SYPA	48	NS	26	46	NS	NS	NS
AT5G14950	Golgi marker	GMII	NS	NS	NS	NS	94	NS	A
AT2G28520	TGN	VHA-A1	NS	NS	50	NS	NS	A	NS

Figure 1. Endomembrane targets. a. Schematic overview of the endomembrane marker proteins used in this study and their localisations. RABD2a/ARA5 – *post*-Golgi/Golgi/TGN/SV, RABF1/ARA6 – LE/MVBs, RABF2b/ARA7 – LE/MVBs, CLC2 – Clathrin Coated Vesicles, GOT1 – Golgi, RABG3f – LE/MVB/Vacuole, VAMP711 – Vacuole. Modified from [16]. **b. Localisation of fluorescent-tagged marker proteins.** Standard confocal micrographs of leaf epidermal cells of *A. thaliana* transgenic plants stably expressing the indicated recombinant proteins. Scale bars 10µM. References: pUB:YFP-RabG3f, YFP-RABD2a/ARA5, YFP-VAMP711, YFP-Got1 [26], RABF1/ARA6-RFP, RFP-RABF2b/ARA7 (provided by K. Schumacher, Heidelberg, Germany), p35S:CLC-GFP (provided by S. Bednarek, Madison WI, USA).

Figure 2. Proteomic workflow and validation. a. Workflow for the affinity enrichment and analysis of endomembrane proteins from *A.thaliana*. b. Immunoblotting of RFP-RABF2b/ARA7 and YFP-RABG3f proteome enrichments to determine organelle contamination. Total protein extracts, from *A.thaliana* Col-0 or stably expressing RFP-RABF2b/ARA7 or YFP-RABG3f, were subjected to immunoaffinity enrichment of RFP or YFP followed by immunoblot with αRFP, GFP, BIP2, COXII, RbcL, AHA1 as indicated.

Figure 3. Sungear diagram of proteins assigned to the different proteomes. Sungear diagrams generated in virtual plant (http://virtualplant-prod.bio.nyu.edu/cgi-bin/sungear/index.cgi [51]). Groups of proteins are indicated by the black dots, with a size proportional to the number of proteins in the group. The arrows on each dot indicate the proteome assignment of a group of proteins. Enrichments were performed three times for each bait, proteins were accepted with SAINT scores >0.8.

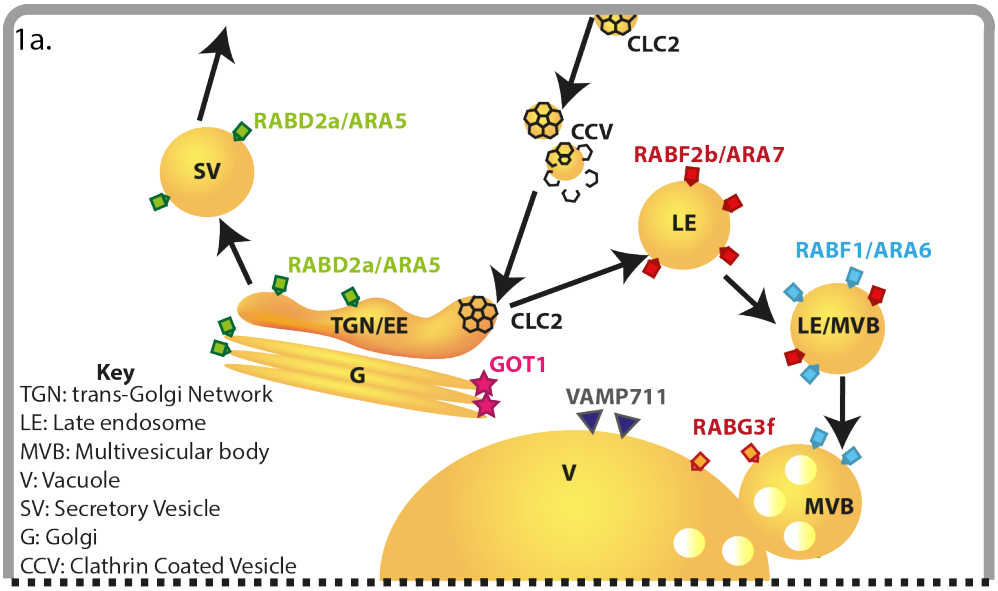
Figure 4. Venn diagrams comparing the proteins assigned to YFP-GOT1 (Golgi) YFP-RABD2a/ARA5 (Golgi/TGN/EE), RFP-RABF2b/ARA7 (LE/MVB) with published endomembrane proteomes. The number in each area of the Venn diagram indicate number of proteins assigned to the proteome or proteomes indicated. Venn diagram comparisons of YFP-GOT1 (Golgi), YFP-RABD2a/ARA5 (Golgi/TGN/EE) and RFP-RABF2b/ARA7 (LE/MVB) proteomes. Proteomes are A - Nikolovski et al.

2012 [39], B - Parsons et al. 2012 [37], C - Sadowski et al. 2008/Dunkley et al. 2006 [33, 53]. D. Drakakaki et al. 2012 [44]. E. Groen et al. 2014 [45]. **a. Comparison of our YFP-GOT1 proteome (Golgi). b. Comparison with our RFP-RABF2b/ARA7 (LE/MVB) and YFP-RABD2a/ARA5 (Golgi/TGN/EE enrichments.**

Figure 5. Venn diagrams comparing the proteins assigned to different endomembrane proteomes. The number in each area of the Venn diagram indicate number of proteins assigned to the proteome or proteomes indicated. **a. Comparison of YFP-GOT1 (Golgi) and RFP-RABF2b/ARA7 (LE/MVB) X^2 – 78.4, reject H_o (of independent assignment of proteins) P<0.001. b. Comparison of RABF1/ARA6-RFP (LE/MVB, YFP-RABG3f (LE/MVB/Vacuole), YFP-VAMP711 (Vacuole).**

Figure 6. Co-localisations of PRA1 family members and RABF1/ARA6 (LE/MVB), RABF2b/ARA7 (LE/MVB) and GOT1 (Golgi). Standard confocal micrographs of leaf epidermis of the indicated *A. thaliana* transgenic plants stably expressing pUB::YFP-GOT1, RFP-RABF2b/ARA7 or RABF1/ARA6-RFP, transiently transformed using particle bombardment, expressing fluorescent tagged PRA1 family members. Insets show an enlarged section of each image.

Figure 1.



1b.

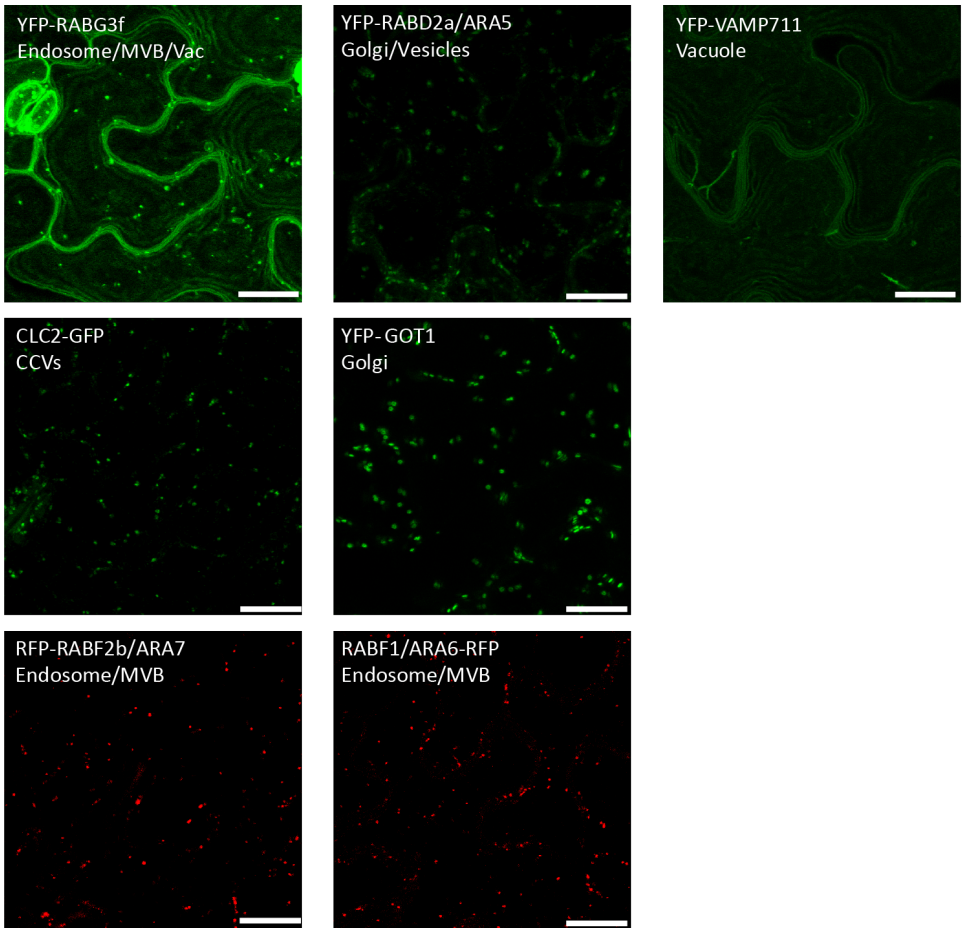


Figure 2.

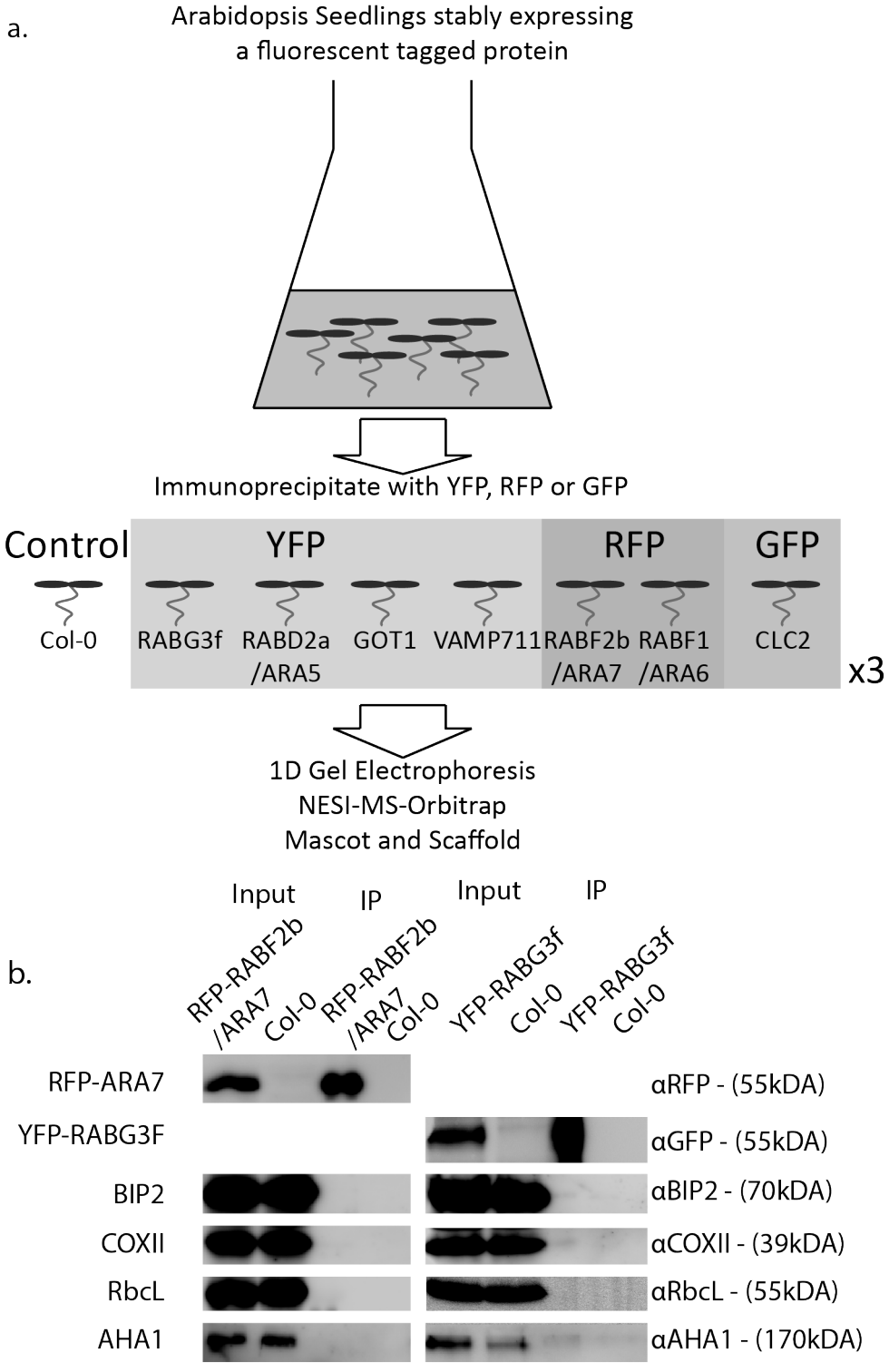
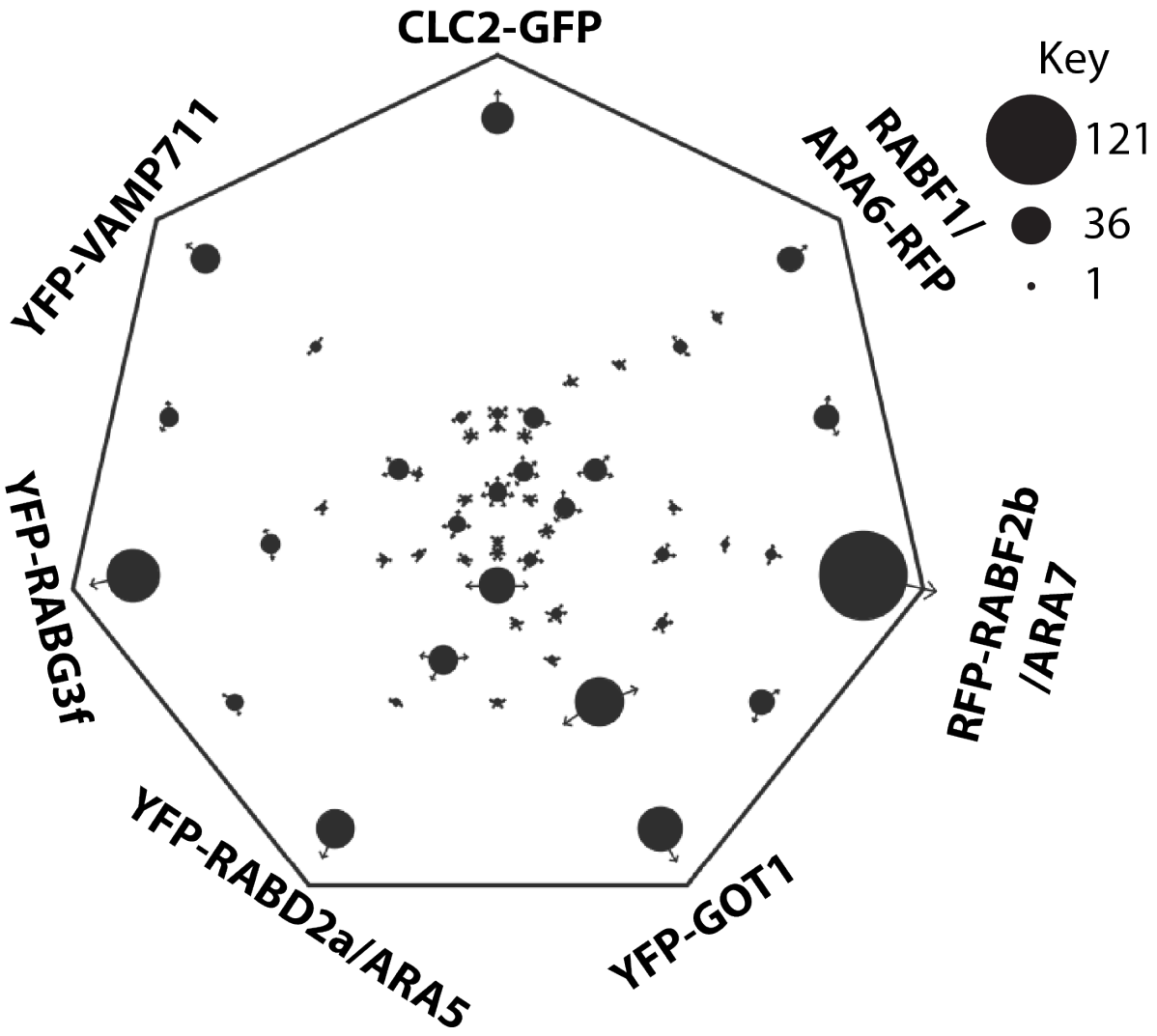


Figure 3.



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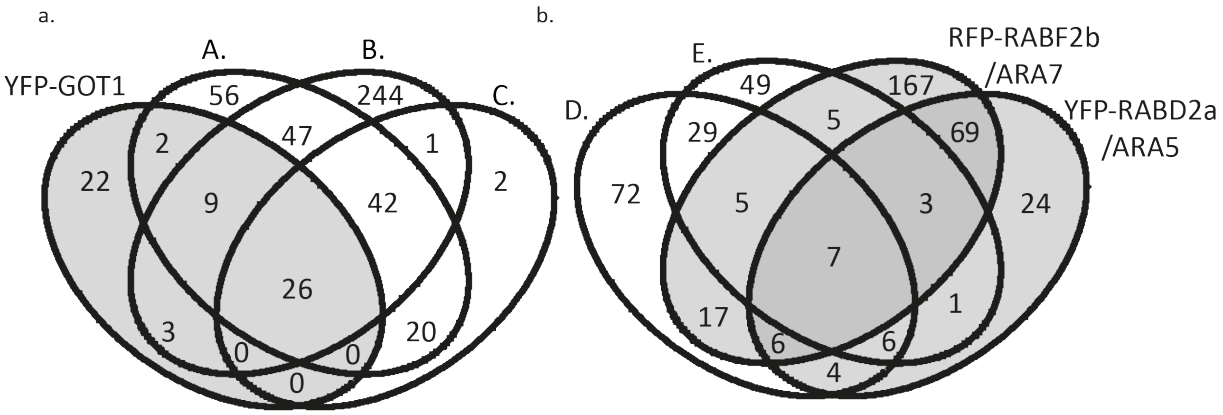


Figure 5.

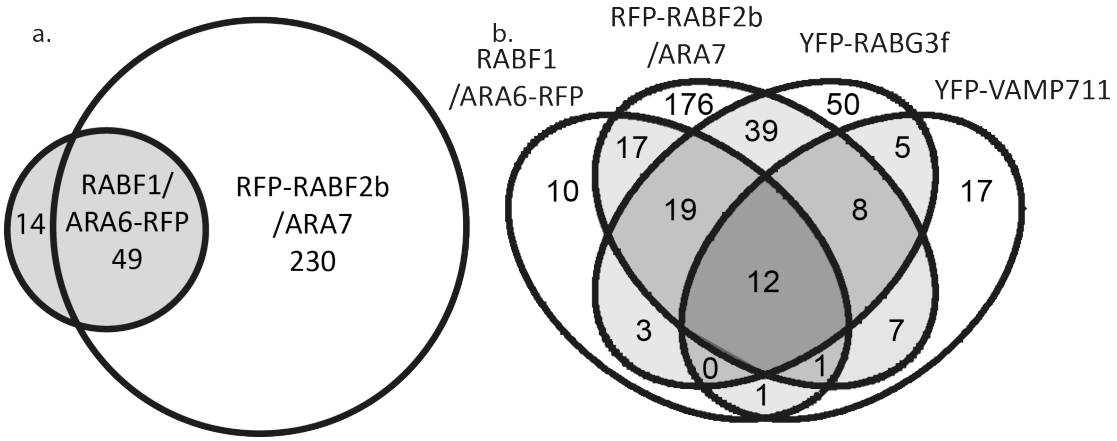
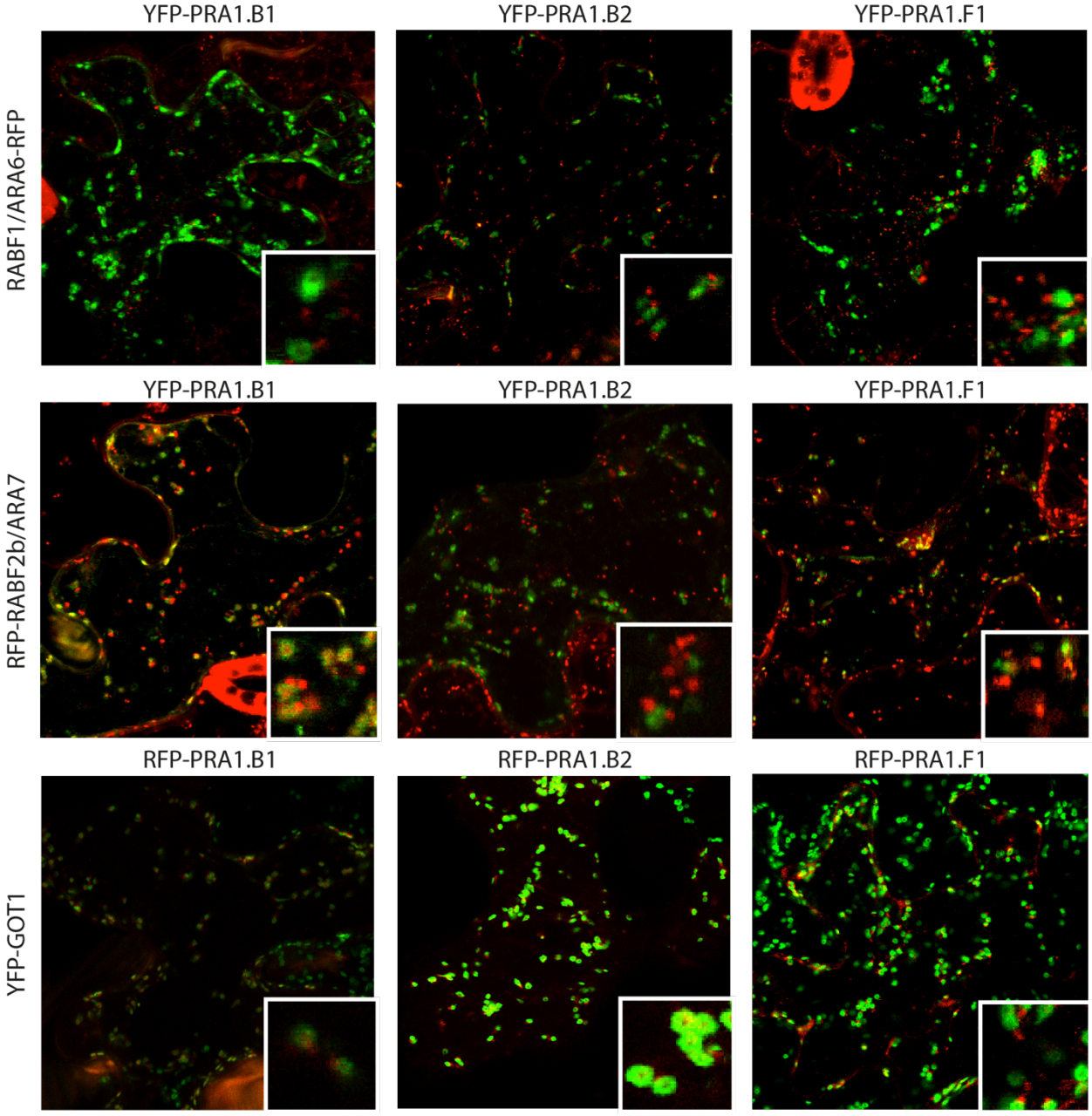


Figure 6.



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